MULTIPLE APPLICATIONS OF 20 HZ ELECTRICAL STIMULATION AFTER PERIPHERAL NERVE INJURY PROMOTE MOTOR AXON REGENERATION AND RETENTION OF EXAGGERATED H REFLEXES

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MULTIPLE APPLICATIONS OF 20 HZ ELECTRICAL STIMULATION AFTER PERIPHERAL NERVE INJURY PROMOTE MOTOR AXON REGENERATION AND RETENTION OF EXAGGERATED H REFLEXES

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LIST OF SYMBOLS AND ABBREVIATIONS

ES	One Time Electrical Stimulation
mES	Multiple Electrical Stimulation
OS	One Time Optical Stimulation
TA	Tibialis Anterior Muscle
GAST	Gastrocnemius Muscle
M response	Direct Muscle Response
H reflex	Monosynaptic reflex

SUMMARY

Exercise promotes regeneration of axons, and a single electrical stimulation can mimic the effect of exercise. A single treatment with one hour of 20 Hz continuous electrical stimulation promotes the regeneration of axons in cut peripheral nerves. This enhanced axon regeneration is the result of a transient increase in neuronal expression of BDNF and its trkB receptor (Al-Majed et al, 2000). A slightly more robust enhancement of peripheral axon regeneration is achieved by two weeks of daily treadmill exercise, also in a BDNF/trkB-dependent manner. We tested the hypothesis that multiple applications of brief electrical stimulation (mES) would be more effective in promoting functional muscle reinnervation than a single application (ES). Sciatic nerves of C57B6 mice were cut and repaired by end-to-end anastomosis. At the time of nerve repair and every third day for the following two weeks, the proximal segment of the cut nerve was stimulated continuously for one hour at 20 Hz. In control groups used for comparison, nerves were cut and repaired and mice were either untreated (UT) or treated with a single application of electrical stimulation (ES) at the time of nerve repair. Beginning two weeks later, functional muscle fiber reinnervation was assayed using stimulus evoked EMG activity from the gastrocnemius and tibialis anterior muscles. Direct muscle (M) responses and monosynaptic H reflexes produced in response to sciatic nerve stimulation above the injury site were studied in awake animals. The amplitude of M responses recorded from reinnervated muscles increased progressively over the 14 week post transection study period. In the mES animals, this increase was more rapid than UT mice but not significantly different from ES mice. In mES mice, the amplitude of H reflexes recorded from

reinnervated muscles increased more rapidly than found in UT mice, but not ES mice, reaching a peak at six weeks after nerve injury. The H reflexes in the mES and ES animals were maintained at more than twice the amplitude of the same reflexes recorded prior to injury for the remaining eight weeks studied. Repeated ES does enhance motor axon regeneration and functional muscle reinnervation, and this enhancement is more robust than a single ES treatment. mES also results in the retention of exaggerated H reflexes.

CHAPTER 1

INTRODUCTION

Every year in the United States, there are about 200,000 new traumatic peripheral nerve injuries. Full functional recovery from these injuries is rare. There is currently no non-surgical treatment for these injuries. One experimental treatment for promoting axon regeneration is exercise (English 2015). Even moderate daily exercise applied after nerve injury induces prolonged high expression of brain derived neurotrophic factor (BDNF) in neurons (Gomez-Panilla et al, 2001), and this expression promotes significant regeneration of injured axons (Wilhelm et al, 2013). However, exercise after peripheral nerve injury and surgical repair might not always be possible. As an alternative to exercise, brief electrical stimulation (ES) of the injured peripheral nerve has proven its ability to promote axonal recovery (Al-Majed et al, 2000a). Most of the time, the treatment has to be given right after one receives a peripheral nerve injury, because delaying application of either exercise or electrical stimulation reduces or eliminates its effect on axon regeneration, elongation and reinnervation (Zhang 2015). Therefore, an alternative to exercise and electrical stimulation is needed.

The most commonly used ES treatment is a single application of one hour of continuous stimulation at 20 Hz at the time of surgical repair of cut nerves. This stimulation produces a transient increase in expression of neuronal BDNF (Al-Majed et al, 2000b) which promotes axon elongation across the surgical repair site (Gordon et al, 2009). Although this single application of ES promotes regeneration of axons across the injury site and into the distal segment of the injured nerve, it is less effective at inducing subsequent elongation of regenerating axons to reach their targets than two weeks of moderate daily exercise

(Sabatier & English, 2015). Based on this difference, I hypothesized that multiple applications of ES might be more effective than a single application. Multiple applications of ES could have an effect in inducing more prolonged expression of BDNF than a single application of ES, and promote axon regeneration/elongation comparable to that observed with exercise.

CHAPTER 2

Methodology

As a peripheral nerve injury model, transection and surgical repair of the sciatic nerve in mice and rats is commonly used both to measure axon regeneration and functional recovery. Mice (C57B6) were chronically implanted with a cuff electrode placed around the right sciatic nerve to be used for stimulation and four pairs of fine wire electromyography (EMG) electrodes to record muscle activity. The EMG electrodes were implanted into the gastrocnemius (GAST) and tibialis anterior (TA) muscles on each side of the animal (Figure 1). All of the wires from these implants were led subcutaneously to a connector plug mounted on the animal's head with dental acrylic (Figure 2). One week after implant surgery, pre-transection (pre-tx) EMG data were collected. In each such recording session, implanted mice were connected to recording amplifiers and an electrical stimulator through the head-mounted connector. In awake animals, the sciatic nerve was stimulated through the implanted cuff electrode, and the EMG activity evoked from the four implanted muscles by that stimulation was recorded and stored on a hard drive. Once pre-tx data had been collected, the mice were anesthetized and

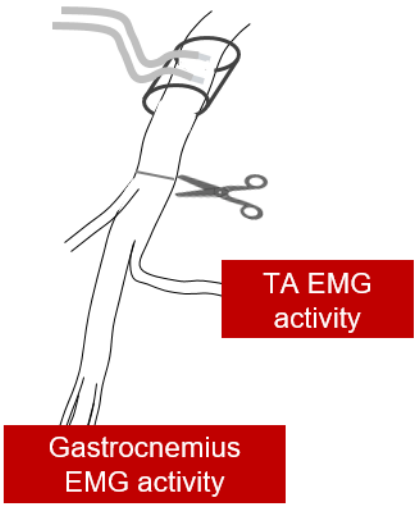

Figure 1: Chronically implanted electrical cuff on sciatic nerve and electrodes from headplug are led to GAST and TA

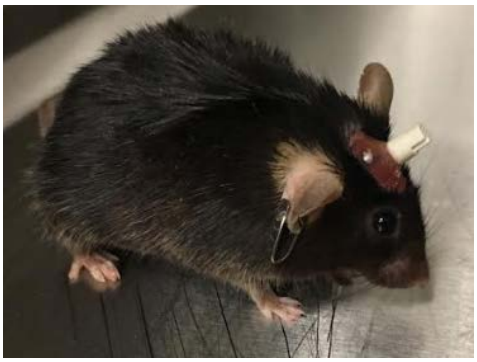

Figure 2: Chronically implanted mouse with a headplug

the sciatic nerve was cut immediately below the cuff electrode and repaired by simple end-to-end approximation using fibrin glue. Electrical stimulation (20 Hz for one hour, 0.3 ms duration pulses) was applied through the implanted cuff electrode to anesthetized animals every 3rd day for two weeks from the transection date. Stimulus intensity during these sessions was set at twice the minimum voltage needed to evoke a muscle twitch in the right GAST muscle prior to nerve injury. Exercise as well as electrical stimulation directly on the injured nerve promotes overexpression of brain derived neurotrophic factor (BDNF). One hour of applied ES results in a rapid increase in expression of neuronal BDNF lasting approximately 72 hours (Al-majed et al, 2000c). Treating with ES every 3rd day for a long period of time is thus an effort to maintain elevated expression of BDNF in sensory and motor neurons. EMG data were collected weekly, as described above, to closely monitor the recovery of innervation of the right GAST and TA muscles and of reflex activity evoked by right sciatic nerve stimulation in the left GAST and TA muscles.

CHAPTER 3

Results

M response

Stimulation of the sciatic nerve produces evoked EMG responses in the ipsilateral GAST and TA muscles. Direct muscle (M) responses are produced by activation of motor axons that innervate muscle fibers in these muscles directly. The amplitudes of the maximal M responses evoked in GAST and TA represent the effect of synchronous activation of all of the motor axons innervating these muscles. When recorded from muscles after injury, this amplitude represents the extent of muscle fiber reinnervation by regenerating motor axons.

The amplitude of the maximal M response in the GAST and TA muscles was measured prior to and at intervals after sciatic nerve transection and repair in mice in three groups: untreated (UT), mice receiving a single treatment with ES at the time of nerve repair (ES), and mice receiving ES treatments every third day for two weeks after nerve repair (mES). In Figure 3, the data in the three different groups were fit with a least squares linear regression. A log transformation was used because the correlation coefficients indicated a better fit of the line to the data than a simple linear fit. Using multiple linear regression analysis, the slopes of these lines for the three different groups were compared using ANOVA. For both GAST and TA, the slopes of the lines for the mice in the ES and mES were significantly ($p<0.05$) greater than that for mice in the UT group, indicating an enhancement of axon regeneration and muscle fiber reinnervation produced by the treatments. When comparing the regression coefficients between ES and mES, no significant difference in either slope or intercept was noted for either muscle. Multiple

electrical stimulation enhanced the regeneration of motor axons no more effectively than a single treatment of ES.

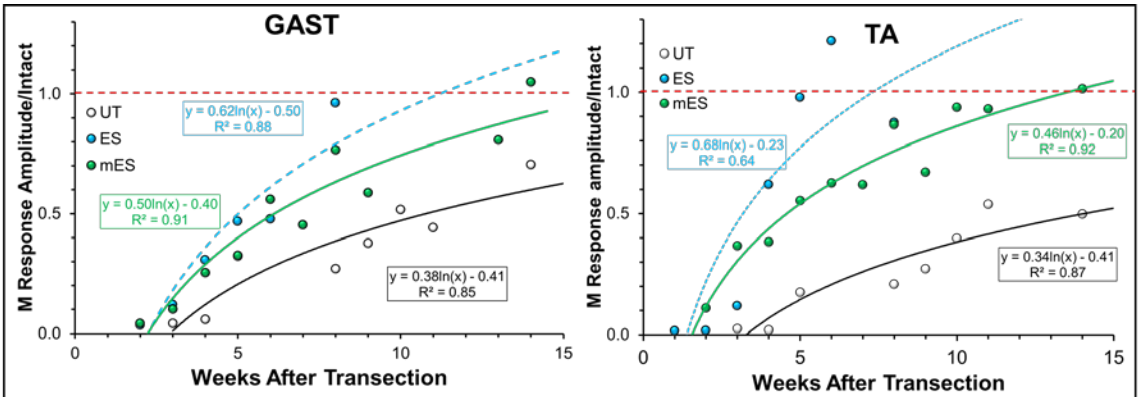

Figure 3: Direct muscle response (M response) of gastrocnemius and tibialis anterior muscles. In each mouse, data obtained from animals after sciatic nerve transection and repair were scaled to its intact M response amplitude. 1.0 in these figures (horizontal dashed lines) represents recovery to 100% of the intact M response amplitude. In gastrocnemius M response, all treatment groups were significant ($p<0.05$) from untreated, but in TA, each treatment was significantly different from each other.

H reflexes

Sciatic nerve stimulation also elicited a second short-latency response known as the H reflex. This muscle response is produced by stimulation of afferent axons in the sciatic nerve that project monosynaptically onto motoneurons innervating either GAST or TA muscles. The largest H reflex amplitudes (Hmax) recorded from muscles after sciatic nerve transection and repair are a measure of the effectiveness of this reflex pathway to the reinnervated muscles. In Figure 4 (top panels), the amplitudes of Hmax recorded from GAST and TA muscles at different times after sciatic nerve transection and repair are shown for the same two treatment groups described above. Data were fit with regression lines, as described above. Slopes of regression lines for the ES and mES groups of both muscles are not significantly different from one another and both are significantly different from that of the UT group of mice, suggesting that both ES and mES had a marked effect on the restoration of this reflex. At all survival times greater than six weeks after sciatic

nerve transection and repair, the amplitude of this scaled H reflex recorded from the reinnervated GAST muscle is significantly larger in ES and mES animals than that found in the UT animals (Figure. 4, lower panels). In contrast, in the TA muscles of these groups of mice, an exaggerated H reflex is found only in the ES animals after 6 weeks. In mice treated with multiple applications of ES, reflex amplitudes had returned to pre-transection levels by six weeks after nerve injury.

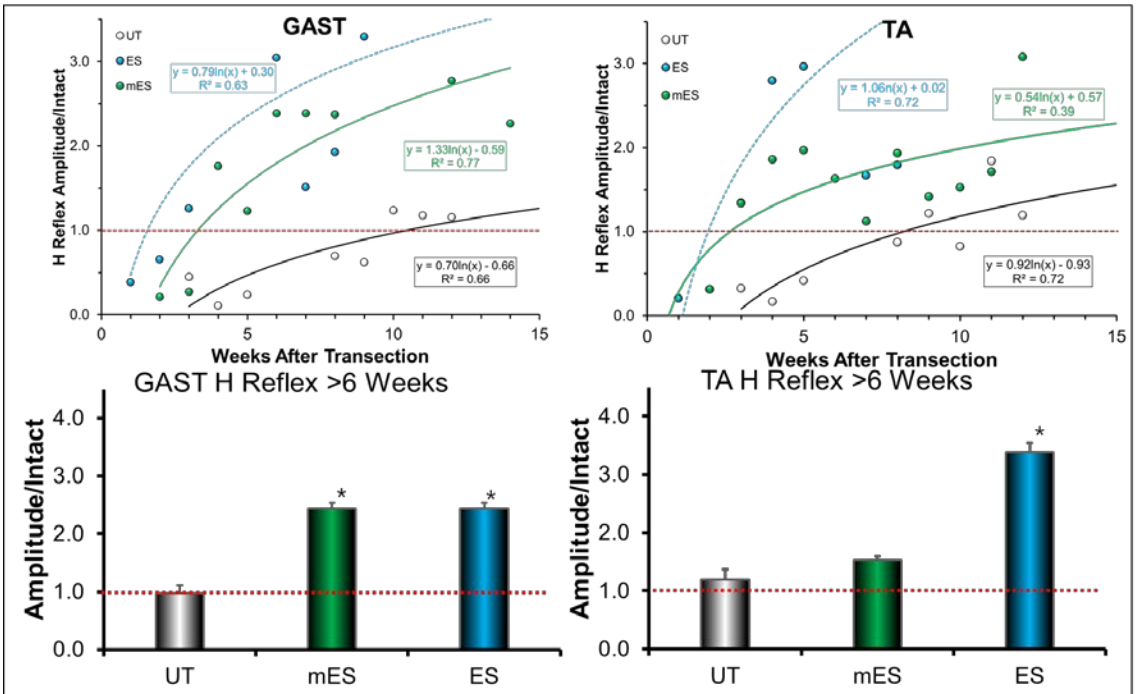

Figure 4: (Top) Monosynaptic H reflexes in gastrocnemius and tibialis anterior muscle. mES and ES significantly ($p<0.05$) enhanced recovery of H reflexes of H reflexes in both GAST and TA. (Bottom) ES had significantly exaggerated H reflexes of GAST and TA after 6 weeks. mES significantly exaggerated H reflex of GAST but exaggerated response was not retained in TA.

Long Latency Reflexes

In the course of these studies, we observed additional responses in both GAST and TA at longer latencies than the M response and H reflex. These typically occur only at stimulus intensities of two times M response threshold or higher. In intact mice, the monosynaptic H reflex is complete by 10 ms after sciatic nerve stimulation but these additional, smaller amplitude repsonses are found between 10 and 50 ms after the end of

the H reflex. Similar, but smaller amplitude responses were found in the left GAST and TA muscles in response to stimulation of the right sciatic nerve. The timing of activity of these long latency responses differed between muscles. A burst of activity in both TA muscles is found bilaterally, beginning at about 15 ms after nerve stimulation in intact mice and lasting approximately 10 ms. At that point, a more prolonged burst of activity is found in both GAST muscles (Figure. 5). In intact mice, these responses were activated reciprocally in GAST and TA.

Among mice in the UT group, these long latency responses were found bilaterlly in reinnervated muscles six weeks after sciatic nerve transeciton and repair (Figure. 6, left panel). Responses evoked in muscles contralateral to sciatic nerve stimulation retained their latencies and amplitudes, as well as their reciprocal timing. In contrast, responses evoked on the ipsilateral side of the animals, from reinnervated GAST and TA muscles at the same post-injury time point, were smaller than noted prior to injury, and the two muscles were activated synchronously, not reciprocally. In mice that had been treated with ES at multiple times, the organization of these responses was different. The reponses in the contralateral limb were barely notable. Two responses were evoked in each of the reinnervated muscles ipsialateral to the sciatic nerve stimulation, and these bursts of activity in GAST and TA were synchronous (Figure. 6, right).

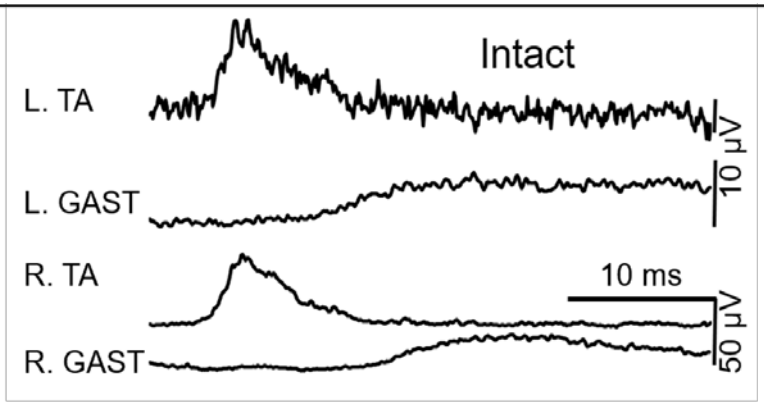

Figure 5: Long latency responses in intact mice. Responses are averaged over 100 trials and six different animals, each at a stimulus intensity of two times M response threshold. Each trace begins at 10 ms after nerve stimulation to avoid demonstration of the larger M response and H reflex in the right muscles. Note that on both sides the timing of the responses of the two muscles is organized in a reciprocal manner.

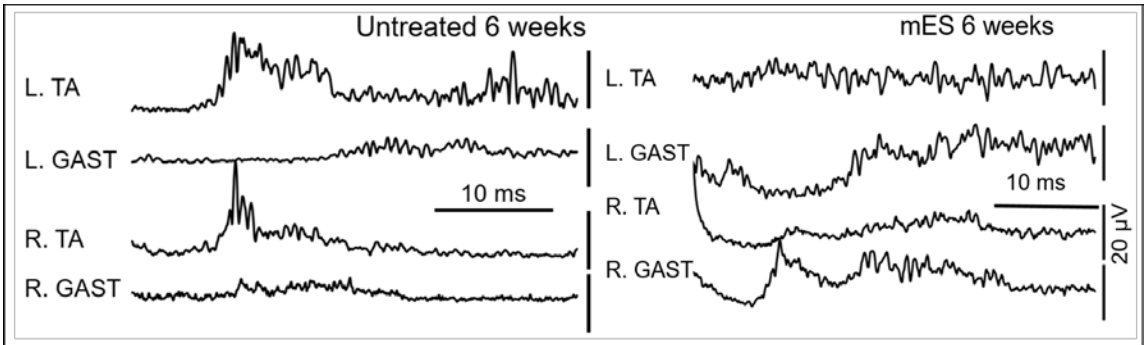

Figure 6: (Left) Long latency responses to right sciatic nerve stimulation at six weeks after right sciatic nerve transection and repair, when untreated. Each trace begins at 10 ms after nerve stimulation to avoid demonstration of the larger M response and H reflex in the right muscles. On the ipsilateral side of the injury (right), both muscles were activated at the same time, whereas on the intact (left) side, they remain reciprocally activated. (Right) Long latency reflexes at 6 weeks when treated with mES. On the ipsilateral side of injury (right), both muscles are synchronically activated and ipsilateral reflexes are not as distinguishable as found prior to injury.

Discussion

The goal of this study was to investigte whether repeated applications of one hour of 20 Hz electrical stimulation (mES) is more effective in promoting axon regeneration after peripheral nerve transection and repair than a single application of this treatment (ES). Our main finding was that M response recovery, a measure of motor axon regeneration and muscle fiber reinnervation, is enhanced about equally after either ES or mES. Similarly, the magnitude of the monosynaptic H reflex recorded from reinnervated muscles in mice treated either with ES or mES was significantly greater than that found in untreated control mice in GAST, and this exaggerated H reflex was retained over a substantial post-injury recovery period.

Both ES and moderate treadmill exercise applied following peripheral nerve injury result in an enhancement of motor axon regeneration (Sabatier & English, 2015), but exercise results in a greater enhancement than ES. We suggest that this difference in effectiveness of these two activity-related therapies might be related to the number of applications in each. Brief ES is applied once, at the time of nerve repair, whereas exercise is applied for short periods, five days per week, for two weeks. It is known that one hour of ES results in a rapid increase in the expression of neuronal BDNF that lasts approximately 72 hours (Al-majed et al, 2000c). Moderate exercise produces a more sustained increase (Sabatier & English, 2015). Because such increases in neuronal BDNF are required for the enhancing effects of ES or exercise on axon regeneration (Wilhelm, 2012), we hypothesized that mES, because it was applied repeatedly, would produce an enhancement of axon regeneration comparable to that observed after exercise. The results of the response data presented above are inconsistent with this hypothesis. Application of

multiple ES to the proximal segment of cut sciatic nerves in GAST did not enhance the regeneration of motor axons and muscle fiber reinnervation any better than a single application. The reason for our failure to promote greater enhancement of motor axon regeneration is not clear at this time. We had assumed that with mES, a more prolonged increase in neuronal BDNF expression would promote greater motor axon elongation. Whether our mES protocol actually produced such a change in BDNF expression remains to be investigated.

We evoked H reflexes from reinnervated muscles by stimulating afferent axons proximal to the injury site and recording the effect on muscle activity. The amplitude of the H reflex thus represents the efficacy with which the stimulated afferent axons recruit motoneurons whose axons have regenerated and reinnervated muscle targets into activity. In untreated control mice, the amplitudes of H reflexes recorded at longer survival times were similar to those recorded from the same muscles prior to sciatic nerve transection and repair, suggesting that the reflex had been restored. In animals receiving one or mutlitple treatments of ES after sciatic nerve transection and repair, the amplitudes of H reflexes at these survival times was markedly larger, at least in GAST, than that of those recorded from the same animals prior to injury. Such exaggerated reflexes have been reported during muscle reinnervation in rats, but they are transient and by 10-15 weeks after injury, the amplitudes of the H reflexes have returned to pre-transection levels or below (Navarro et al. 2007; Boeltz et al. 2013). Our finding that these exaggerated H reflexes persisted in mice that had been treated with ES or mES in GAST and only ES in TA is interpreted as evidence that whatever the mechansim of decline in amplitude of the exaggerated H reflexes noted in untreated control mice was blocked by our activity-dependent treatments

applied shortly after nerve injury. It will be of considerable interest to invetigate the changes in neural circuitry that might contribute to this persistent effect. It is also notable that the amplitude of the H reflexes recorded from reinnervated muscles in exercised rats are not exaggerated (Boeltz, 2013). In terms of the recovery of the H reflex, treatments with mES and ES do not replicate those found with exercise.

During the testing of mice for muscle reinnervation and H reflex magnitude, a novel muscle response was observed bilaterally. The response appeared at a much longer latency than either the M response or the H reflex and, at least in intact animals it was characterized by short bursts of activity that were timed reciprocally in GAST and TA. It is not entirely clear whether these responses, that were evoked only from stimulation of smaller diameter afferent neurons, were polysynaptic segmental reflexes or involved a longer reflex loop, but they were observed in all animals studied. In reinnervated muscles, regardless of post-inury treatment regimen, bursts of activity in GAST and TA at the appropriate latencies occurred synchronously, regardless of the treatment that had been applied to the animal. In untreated mice, responses recorded from the contrateral muscles remained and the timing in GAST and TA was reciprocal. In mES mice, responses from the contrateral muscles did not appear.

The reason for the change in activation sequnces in reinnervated muscles could be explained by misdirection of axons reinnervating the muscle. After the sciatic nerve is cut, some motoneurons that innervated GAST could be misdirected during regeneration and reinnervate TA and vice-versa. If these motoneurons were recruited into these long latency responses in a manner similar to that found in intact mice, the result would be a co-activation of GAST and TA, as was noted. However, the near complete loss of these

responses in the contralateral GAST and TA muscles in mice in the mES group could not be as easily explained. Changes in the central circuitry that underly these long latency responses must be considered. Precise distinction and how much each of these two sources of change contributes to recovery could be tested in future studies using more precise muscle reinnervation surgeries.

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